[Chem. Pharm. Bull. 23(8)1806—1813(1975)]

UDC 547.596.5'233.03 : 543.51'544

Mass Fragmentographic Determination of *trans*-4-Aminomethylcyclohexanecarboxylic Acid (Tranexamic Acid) by Use of Peak Matching Operation

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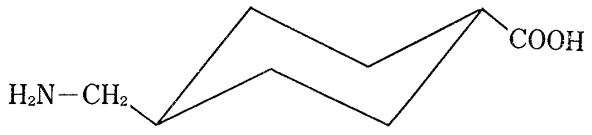
(Received December 26, 1974)

The concentration of *trans*-4-aminomethylcyclohexanecarboxylic acid (tranexamic acid) in human serum was determined by peak matching operation. N-Heptafluorobutyryl amide derivative of tranexamic acid *n*-butyl ester was found to be the most suitable derivative for this purpose. The present operation enabled to confirm that peak due to tranexamic acid had no contaminants at all. Deuterium labeled tranexamic acid was used as an internal standard for quantiation of tranexamic acid in human serum. It was also found that proteins in human serum were precipitated completely with heptafluorobutyric acid which was readily removed by evaporation. On the oral administration of 250 mg of tranexamic acid, the serum level concentration reached maximum at 3—4 hour post-dose period.

Introduction

Tranexamic acid (*trans*-4-aminomethylcyclohexanecarboxylic acid) has been found out to be effective for various diseases associated with increased plasmin activity because of its strong anti-plasmin activity.[2] Microdetermination for tranexamic acid in biological fluids has been carried out by colorimetry[3] and gas chromatography,[4] but these methods are lacking in the specificity of detection. In order to determine trace amount of tranexamic acid in the biological samples by these methods, therefore, it is necessary to confirm the absence of any interfering substances in the specimen.



tranexamic acid (*trans*-4-aminomethylcyclohexanecaboxylic acid)

Hence, development of more specific, accurate and sensitive method is of great importance for investigating pharmacodynamics and metabolism of tranexamic acid.

Mass fragmentography developed by Sweeley, *et al.*[5] is one of the most suitable methods to achieve the above purpose. Since an internal standard labeled with stable isotope has been utilized in mass fragmentographic analysis,[6] great many papers on quantitative determination of drugs and endogenous substances using this technique have been reported as reviewed by Gordon and Frigerio,[7] Jenden and Cho,[8] Holmstedt and Palmer,[9] and Burlingame, *et al.*[10]

1) Location: *3-31 Shimo, Kita-Ku, Tokyo, 115, Japan.*
2) S. Okamoto and U. Okamoto, *Keio J. Med.*, **11**, 105 (1962).
3) Y. Takata, A. Tanaka, and U. Okamoto, *J. Physiol. Soc. Japan.* **26**, 281 (1964).
4) H. Kitajima, M. Hirakawa, and M. Ichinose, *Iyakuhin Kenkyu*, **2**, 34 (1971).
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9) B. Holmstedt and L. Palmer, in E. Costa and B. Holmstedt(Eds), *Adv. Biochem. Psychopharmacol.*, **7**, 1 (1973).

On the other hand, for discriminating trace amount of drug from great quantities of endogenous substances in biological fluids, it has attracted attention in the field of clinical pharmacology to monitor molecular and/or fragment ion clusters stemmed from the drug in mass fragmentography.[11–13] The peaks assignable to the drug on mass fragmentogram were identified by monitoring simultaneously two characteristic fragment ions, the intensities of which were evened up previously by adjusting a gain controller in a multiple ion detector. The principle of this technique has been described by Hammar and Hessling,[14] but its biomedical application has not been published yet. The identification by this technique is very similar to that by 1:1 mixture technique which involves an artificially created ion cluster as a useful marker.

In this paper, this technique was applied to the determination of tranexamic acid in human serum with use of deuterium labeled tranexamic acid as an internal standard for enhancing the reliability of the determination.

Experimentals

Mass Spectrometry and Mass Fragmentography——Shimadzu-LKB 9000 GC-MS systems equipped with accelerating voltage alternator (LKB-9066), multiple ion detector (LKB-9060) and data processing system (Shimadzu GCMSPAC-300) were employed. The column was 2 m×3 mm i.d., glass coil with 3% OV-3 (Ohio Valley Co., U.S.A.) on Chromosorb W-HP 80—100 mesh (Johns-Manville Co., U.S.A.). The temperature of column oven was maintained at 185°. The flow rate of carrier gas (Helium) was 30 ml/min. The temperature of injection port and separator was 210°, and ionization source was kept at 250°. Accelerating voltage was 3500 V. The ionization energy and trap current were 20 eV and 60 μA, respectively.

Samples and Reagents——All reagents and solvents used in this research were of analytical grade and were used without further purification.

Tranexamic acid used as standard material was obtained through several recrystallizations of technical grade. *cis*-4-Aminomethylcyclohexanecarboxylic acid was prepared from ethyl *p*-acetamidomethylbenzoate by the method of Naito, *et al.*[15] Tranexamic acid-d_6 (*trans*-4-aminomethylcyclohexane[d_6]carboxylic acid) and *cis*-4-aminomethylcyclohexane[d_6]carboxylic acid as internal standards were prepared according to the above method using deuterium gas instead of hydrogen gas in hydrogenation process. *trans* and *cis*-4-N-Heptafluorobutyrylaminomethylcyclohexane[d_6] carboxylic acid butyl esters were prepared from *trans* and *cis*-4-aminomethylcyclohexane[d_6]carboxylic acids by esterification with dry hydrogen chloride–*n*-butanol solution and amidation with heptafluorobutyric anhydride.

Heptafluorobutyric anhydride was prepared from heptafluorobutyric acid (Pierce Chemical Co., Rockford, Illinois U.S.A.), and distilled on phosphoric anhydride according to the method of Sawicks.[16]

Procedure——The volunteers of healthy adult male were administered with a single oral dose of 0.25 gram of tranexamic acid. Blood specimens were taken at 1, 2, 3, 4, 6, and 8 hours after administration.

To 1 ml of serum in 10 ml centrifuging tube, 1.0 ml of internal standard solution (4.5 μg tranexamic acid-d_6/ml), 3 ml of water were added and mixed. Then, 100 μl of heptafluorobutyric acid were added gradually. The tube was shaken well by hand and allowed to stand for 10 minutes, then centrifuged for 15 minutes at 2000 rpm. Two ml of supernatant was evaporated under reduced pressure. After adding 0.5 ml of *n*-hexane to the residue, 0.5 ml of heptafluorobutyric anhydride was added under cooling in ice-bath, then the mixture was heated at 60° for 5 minutes. To the reaction mixture, 0.5 ml of heptafluorobutyric anhydride was added again and 0.5 ml of *n*-butanol was gradually added along the inner wall of flask. After heating at 60° for 10 minutes, the reaction mixture was evaporated under reduced pressure. The residue was extracted with 1 ml of ethyl acetate, and the extract was transferred into 5 ml of centrifuging tube. This extraction procedure was repeated three times. The ethyl acetate layers were gathered in the above tube, washed with 1.5 ml of water, and centrifuged at 2000 rpm for 5 minutes. The supernatant was transfered into another tube, dried over anhydrous sodium sulfate, and concentrated to approximately 0.1 ml. This concentrate was chromatographed over silica gel-column (0.9×5 cm, activated at 130°, 15 hours) in *n*-

10) A.L. Burlingame, R.E. Cox, and P.J. Derrick, *Anal. Chem.*, **46**, 248R (1974).
11) T.E. Gaffney, C-G. Hammar, B. Holmstedt, and R.E. McMahon, *Anal. Chem.*, **43**, 307 (1971).
12) M. Inoue, M. Ito, M. Ishibashi, and H. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), **22**, 1949 (1974).
13) H. Miyazaki, M. Ishibashi, T. Izawa, H. Takayama, and G. Idzu, *Chem. Pharm. Bull.* (Tokyo), **23**, 837 (1975).
14) C-G. Hammar and R. Hesslimg, *Anal. Chem.*, **43**, 293 (1971).
15) T. Naito, S. Okano, S. Kadoya, and T. Miki, *Chem. Pharm. Bull.* (Tokyo), **16**, 728 (1968).
16) E. Sawicks, *J. Org. Chem.*, **21**, 376 (1956).

hexane–ether (85 : 15). The first 50 ml of eluate was collected and concentrated to approximately 1 ml, then the concentrate was provided for the mass fragmentographic analysis of tranexamic acid.

Result and Discussions

Derivatization

One step derivatization methods used in gas chromatographic analysis of amino acids were applied to tranexamic acid, but the results were far from satisfaction with stability and reproducibility.

On the other hand, alkyl ester-N-acyl amide derivative, which is widely used in amino acid analysis, gave a very excellent result also in the case of microdetermination of tranexamic acid. In order to avoid the interference of other low molecular weight materials in extract, the base peak of this derivative should be shifted to as high mass region as possible. In addition, it is necessary for peak matching operation that the above base peak is followed by a

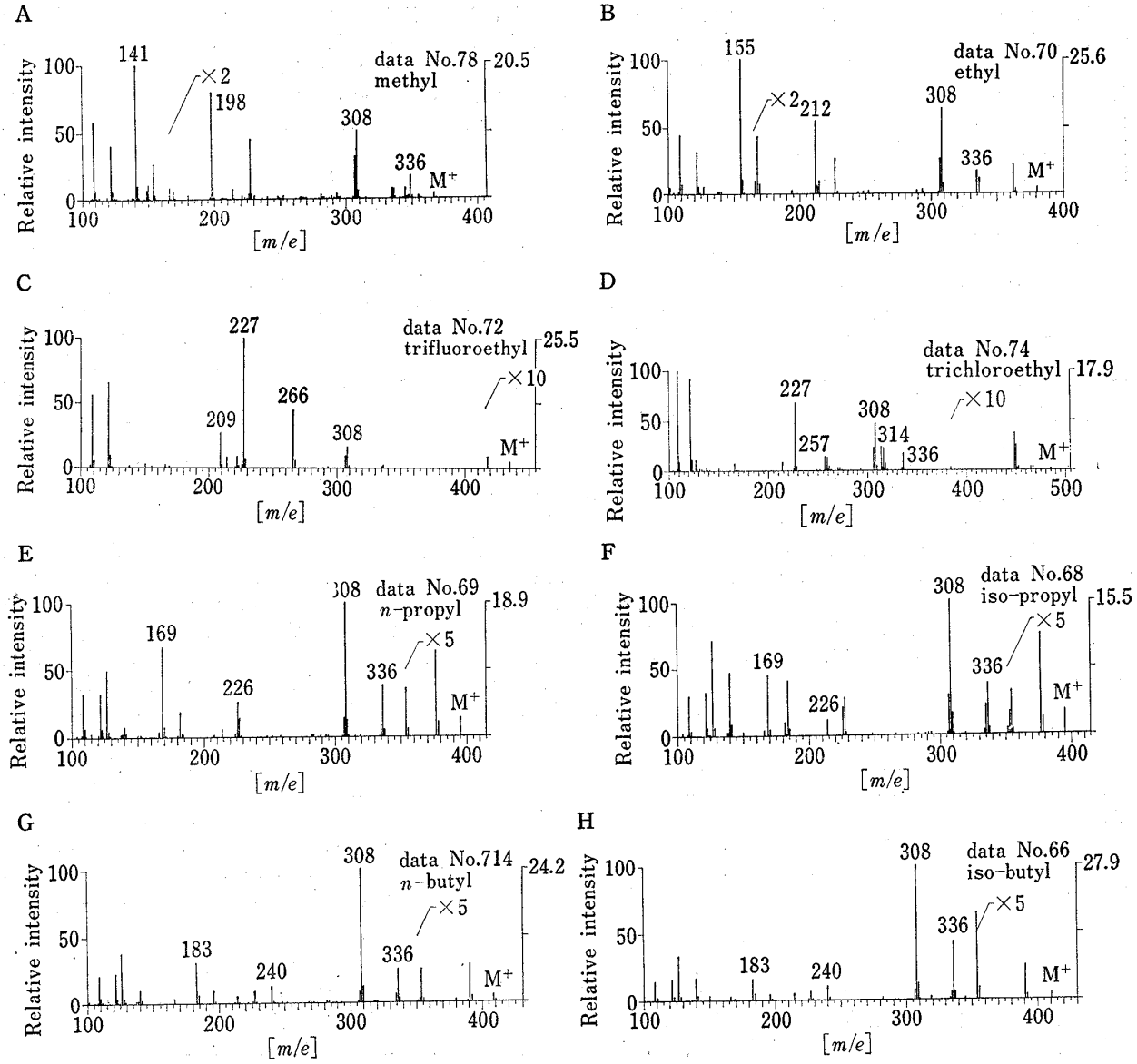

Fig. 1. Mass Spectra of (A) Methyl, (B) Ethyl, (C) Trifluoroethyl, (D) Trichloethyl, (E) *n*-Propyl, (F) Iso-propyl, (G) *n*-Butyl and (H) Iso-butyl Ester-N-Heptafluorobutyryl Amide Derivatives of Tranexamic Acid

characteristic fragment ion with abundant intensity within a mass range limitation of multiple ion detector.

On detailed examination of mass spectra due to methyl, ethyl, trifluoroethyl, trichloroethyl, *n*-propyl, isopropyl, *n*-butyl and iso-butyl ester -N-heptafluorobutyryl amide derivatives of tranexamic acid, *n*-butyl ester was found to be most satisfactory to the above mentioned criteria. Mass spectra of the above derivatives and the mass fragmentation of *n*-butyl derivative are shown in Figures 1-A to 1-H and 2, respectively.

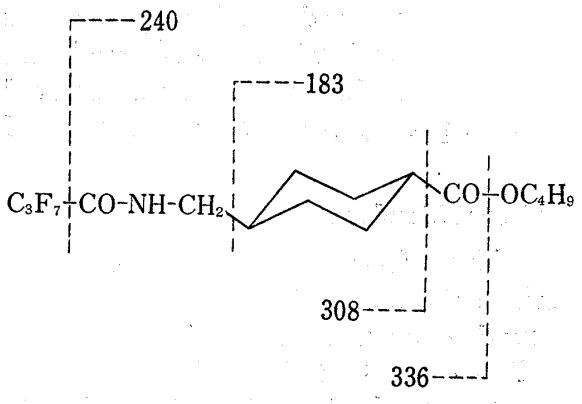

Fig. 2. Mass Fragmentation of *n*-Butyl Ester-N-Heptafluorobutyryl Amide Derivative of Tranexamic Acid

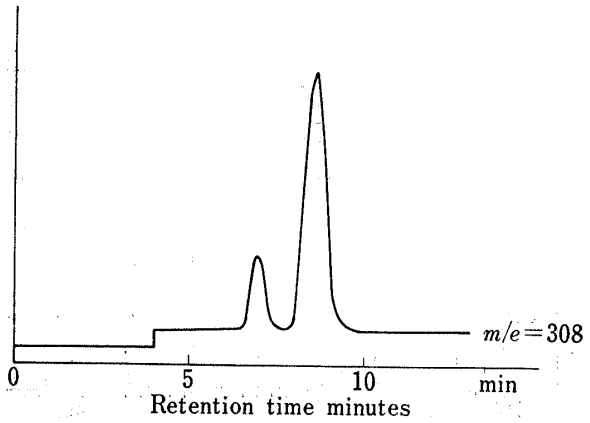

Fig. 3. Mass Fragmentogram of Reaction Product obtained by Derivatization of Tranexamic Acid

The derivatization with dry hydrogen chloride–*n*-butanol and heptafluorobutyric anhydride gave a desirable result for authentic tranexamic acid. However, when this procedure was applied to the extract of biological fluids, the satisfactory results could not be obtained because the esterification in this case was incomplete. Thus, various methods have been examined for esterifying tranexamic acid in extract and satisfactory result was ensured by improving Smith's method.[17] This derivatization made it possible to carry out an amidation and an esterification of tranexamic acid within a short time.

When this reaction mixture was subjected to mass fragmentography, an unexpected minor peak appeared on the mass fragmentogram as shown in Figure 3. However, it was found that

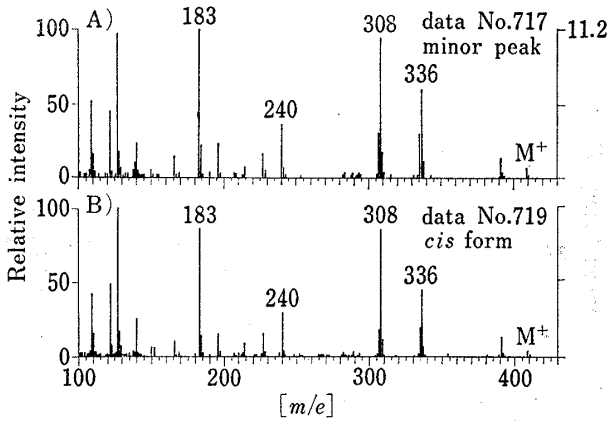

Fig. 4. Mass Spectra of (A) Minor Product obtained by Derivatization and (B) *n*-Butyl Ester-N-Heptafluorobutyryl Amide Derivative of *cis*-4-Aminomethylcyclohexanecarboxylic Acid

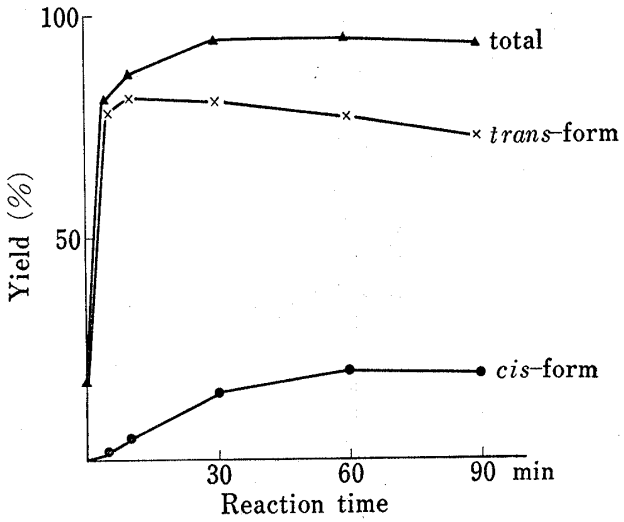

Fig. 5. Time Course of *trans-cis* Conversion on First Step Reaction

17) R.V. Smith and S.L. Tsai, *J. Chromatogr.*, **61**, 29 (1971).

GC-MS spectrum of this peak was identical to that of authentic sample of *cis*-4-aminomethylcyclohexanecarboxylic acid butyl ester (Fig. 4). This may be due to the tranformation of *trans* to *cis*-form during amidation reaction. The time course investigation of the *trans-cis* conversion reaction was carried out by mass fragmentography using *trans*- and *cis*-4-N-heptabutyrylaminomethylcyclohexane[d_6]carboxylic acid methyl esters as internal standards. After an aliquot of standard tranexamic acid solution was added to serum, the tranexamic acid was extracted, purified and derivertized according to the same manner as described in the experimental.

Thus, amounts of *trans* and *cis*-forms in the reaction mixture was determined by the mass fragmentography. From the above mentioned time couse investigation, it was found that *cis*-form was produced only at the amidation step. Also, most of the conversion was observed at the early stage of derivatization as shown in Fig. 5. Eightly percents of tranexamic acid remained in *trans*-form within 5 min amidation.

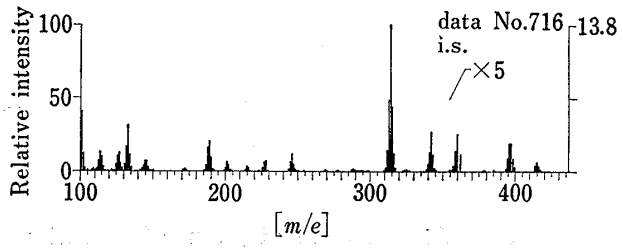

Fig. 6. Mass Spectrum of *n*-Butyl Ester-N-heptafluorobutyryl Amide Derivative of Multi-deuterated Tranexamic Acid Mixture

Internal Standard

Mass fragmentography enables to utilize the stable isotope labeled compound as an internal standard, and it is indispensable that the stable isotope in internal standard must be located onto the fragment ions which are monitored for quantitative analysis. Peak matching operation work was achieved through simultaneous monitoring of the base peak (*m/e* 308) and characteristic fragment ion (*m/e* 336), both containing a cyclohexane ring. Then, deuterium labeling was done into cyclohexane ring for preparing an internal standard.

Mass spectrum of deuterated tranexamic acid is shown in Fig. 6. Mass spectrometric determination of deuterium content in this internal standard (i.s.) is shown in Table I. These results suggest that internal standard is a mixture of multi-deuterated isomers. The isotopical purity of hexadeuterated tranexamic acid, the main component in the internal standard, was calculated to be about 46%. To determine the maximum permissible amounts of the

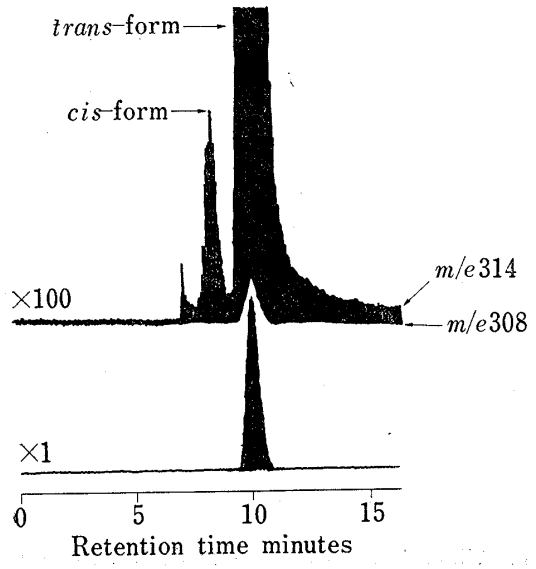

Fig. 7. Recording of Ion Intensities at *m/e* Value 308 and 314 after Injection of Internal Standard

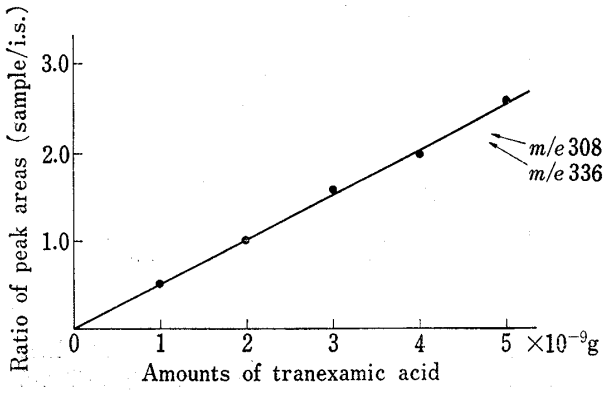

Fig. 8. Calibration Curve of Tranexamic Acid

Table I. Mass Spectrometric Analysis of Deuterium Content in Internal Standard

m/e	Relative intensity	Deuterium content (%)
308(d_0)	—	less than 0.1
209(d_1)	—	less than 0.1
310(d_2)	1.1	1.2
311(d_3)	3.0	3.0
312(d_4)	14.7	15.2
313(d_5)	49.5	50.6
314(d_6)	100.0	100.0
315(d_7)	44.7	35.4
316(d_8)	12.5	9.0
317(d_9)	2.6	1.7

internal standard added into serum, the accurate ratio of unlabeled tranexamic acid (d_0-compound) and tranexamic acid-d_6 in the internal standard was determined by the mass fragmentography. Fig. 7 shows the recording of ion intensities at m/e values 308 and 314. This mass fragmentogram suggests that the ratio of d_0-compound to tranexamic acid-d_6 is less than 0.3%, and also this fact indicates that it is permissible to add the internal standard to the extent of 10 times of quantity of tranexamic acid to serum.

Deprotenization

Deprotenization is inevitable to mass fragmentographic analysis of drugs in biological specimens such as blood and organ homogenates. Trichloroacetic acid is widely used for this purpose. But, the use of this reagent needs tedious procedures for extraction, cleaning up and derivatization because of difficulty in the elimination of the excess acid after deprotenization. In responce to the problem, heptafluorobutyric acid which exhibits strong acidity and very high volatility was found to have the same deprotenizing effect as trichloroacetic acid and to be easily removed by evaporation.

As the supernatant after deprotenization was used for derivatization of tranexamic acid without further purification, it was indispensable to carry out a clean up procedure for reaction products. Then, silica gel column chromatography was applied for elimination of other interfering substances, but the derivative of *cis*-4-aminomethylcyclohexanecarboxylic acid was not separated by this operation.

Calibration Curve

The calibration curve for tranexamic acid, plotting peak area ratio of tranexamic acid to the internal standard (tranexamic acid-d_6) against amounts of tranexamic acid added to serum, is illustrated in Fig. 8. Linear relationship is obtained in the range of 1 to 5×10^{-9} gram.

Reliability

Knapp, *et al.* have developed the artificially created ion cluster technique[18] to detect a drug and its metabolites in biological sample.

In this study, two characteristic fragment ions (m/e value 308 and 336) were picked up and the doublet with equal intensity was created mechanically from these ions by adjusting a gain controller in multiple ion detector. Then, doublet is monitored simultaneously in much the same way as artificially created ion cluster technique as shown in Fig. 9. Thus, the present technique may provide high reliability as well as the ion cluster technique to analytical results.

18) D.R. Knapp, T.E. Gaffney, and R.E. McMahon, *Biochem. Pharmacol.*, **21**, 425 (1972).

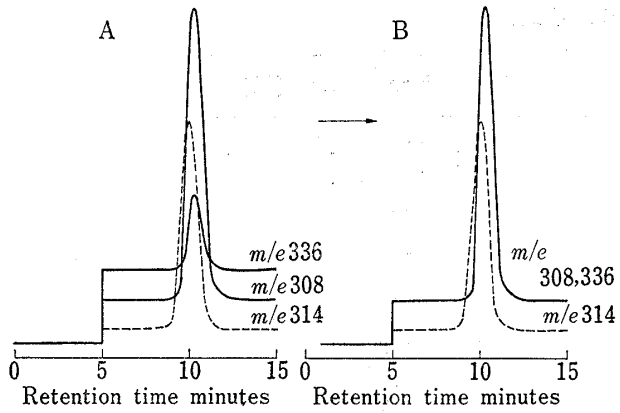

Fig. 9. Mass Fragmentograms of *n*-Butyl Ester-N-Heptafluorobutyryl Amide Derivative of Tranexamic Acid recorded by (A) Conventional and (B) Peak Matching Operations

When the purified sample was subjected to this simultaneous monitoring system, peaks of two fragment ions were recorded with almost same peak heights and peak shapes on mass fragmentogram. The ratios of peak areas of *m/e* value 336 to 308 were measured on the authentic compound and the extract from serum. As shown in Table II, satisfactory results were obtained by the present technique. These results suggest that the peaks corresponding to derivative of tranexamic acid have no contaminants at all.

The serum level concentrations of tranexamic acid could be determined by the present technique without overestimation.

Accuracy

Accuracy of mass fragmentographic determination for serum levels of tranexamic acid was studied. One microgram of tranexamic acid and five-fold weight of internal standard (as tranexamic acid-d_6) were added to serum. Tranexamic acid was extracted, purified, derivatized and analyzed in the same manner as described in the experimentals. The analytical data and recoveries are shown in Table III. Coefficient of variation of these recoveries gave 5% for 1 microgram of tranexamic acid per 1 ml of serum. As the error by losses of tranexamic acid in the extraction, clean up, and derivatization operations can be compensated by use of the internal standard labeled with stable isotope, most of the error in this determination seems to be attributable to the operation by mass fragmentographic analysis.

TABLE II. Reproducibility of Ratio of Peak Areas with *m/e* Value 336/308 for Standard and Biological Samples

Sample		$(336/308)_{AREA}$	Mean±SD
Volunteer	1	1.019	
		0.953	
	2	0.998	
		0.937	
	3	1.092	1.012±0.061
		0.940	
	4	1.066	
		0.971	
	5	1.059	
		1.087	
Standard material		1.017	
		1.089	
		1.021	
		0.971	1.022±0.056
		1.027	
		1.091	
		0.939	

Table III. Recovery of Added Tranexamic Acid

Added amount (μg/ml)	Found amount (μg/ml)	Recovery (%)	Mean±SD
1.000	1.057	105.7	1.004±0.052
	1.033	103.3	
	0.921	92.1	
	0.988	98.8	
	1·019	101.9	

Quantitative Analysis for Serum Level Concentration of Tranexamic Acid

Characteristic fragment ions of tranexamic acid and tranexamic acid-d_6 were used for quantitative analysis. Peak corresponding to tranexamic acid could be identified by using the informations on the fragment ions and their relative intensities in combination with the gas chromatographic retention time. And also, it was verified by peak matching operation that the objective peaks had no contaminants due to biological substances and gave accurate analytical results without any overestimation.

The serum level concentration of tranexamic acid is shown in Fig. 10, which indicates maximum point at 3 to 4 hour post-dose period at the level to 5 microgram per 1 ml followed by the monotonic decrease with a half-life of 2.15 hour (Fig. 10).

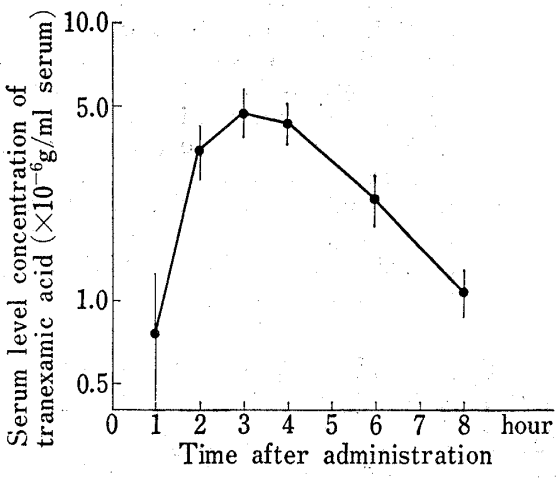

Fig. 10. Serum Level Concentration of Tranexamic Acid in Five Healthy Adult Men dosed Single 0.25 gram

Acknowledgement The authors are grateful to W. Tanaka, Nippon Kayaku Co., for his encouragements and valuable suggestions through this work.